

Tumor budding in colorectal carcinoma assessed by cytokeratin immunostaining and budding areas: Possible involvement of c-Met

Keisuke Satoh,^{1,2} Satoshi Nimura,¹ Mikiko Aoki,¹ Makoto Hamasaki,¹ Kaori Koga,¹ Hiroshi Iwasaki,¹ Yuichi Yamashita,² Hiroaki Kataoka³ and Kazuki Nabeshima¹

Departments of ¹Pathology and ²Gastroenterological Surgery, Fukuoka University School of Medicine and Hospital, Fukuoka, Japan; ³Section of Oncopathology and Regenerative Biology, Department of Pathology, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan

Key words

tumor budding, sprouting, c-Met, tumor invasion

Correspondence

Kazuki Nabeshima, Department of Pathology, Fukuoka University School of Medicine and Hospital, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan.
Tel: +81-92-801-1011; Fax: +81-92-863-8383;
E-mail: kaznabes@fukuoka-u.ac.jp

Funding information

Ministry of Education, Culture, Sports, Science, and Technology, Japan; Research Center for Advanced Molecular Medicine, Fukuoka University, Japan

Received May 20, 2014; Revised August 20, 2014;
Accepted August 29, 2014

Cancer Sci 105 (2014) 1487–1495

doi: 10.1111/cas.12530

Tumor budding/sprouting has been shown to be an independent adverse prognostic factor in T1 and T3N0 colorectal carcinomas, however, its assessment could be improved by more accurate identification of budding carcinoma cells and consideration of budding areas. Moreover, tumor budding mechanisms are yet to be defined. In this study, we evaluated the identification of budding tumor cells by either H&E staining alone or H&E with immunohistochemistry and developed a scoring system based on budding grades and areas. We examined whether the budding score correlated with clinicopathologic features and prognosis and the association between tumor budding/sprouting and c-Met protein expression and phosphorylation and *MET* gene copy numbers because c-Met is known to play an important role in colorectal carcinoma tumorigenesis. Cytokeratin immunohistochemistry could identify tumors with shorter disease-free survival (DFS) from the low-grade budding group assessed with H&E alone. High budding scores based on budding grade and area were more significantly correlated with DFS than scores obtained using the budding grade alone. In tumors with a high budding score, c-Met expression and phosphorylation levels and *MET* gene copy numbers were significantly increased at the invasive front compared with those in superficial tumor portions. This study showed for the first time that high levels of phospho-c-Met at the invasive front were significantly associated with a high budding score and shorter DFS. In conclusion, a budding score assessed by budding grades and budding-positive areas correlates highly with clinicopathologic aggressive features of colorectal carcinoma.

Colorectal carcinoma is one of the most common solid tumors worldwide.⁽¹⁾ Clinicopathologic staging of colorectal tumors is carried out for prognosis and treatment decision using the TNM staging system from the Union for International Cancer Control.⁽²⁾ However, a substantial number of tumors demonstrate local or distant recurrences despite being categorized as low risk by the TNM system.^(3,4) The failure of TNM staging to serve as a reliable prognostic system for patients with intermediate-stage tumors may be overcome by considering morphologic, molecular, or treatment-related factors that can stratify patients more precisely into different categories of risk.⁽⁵⁾ Thus, the search for additional factors to assess the prognosis of colorectal carcinomas has been a major research focus. The promising histopathologic parameters in colorectal carcinoma include extramural venous invasion, tumor border configuration (pushing vs. infiltrating), presence of an inflammatory infiltrate, and tumor budding/sprouting, defined as small clusters of tumor cells at the invasive front.⁽³⁾

The concept of tumor budding/sprouting was first described in the 1950s by Imai, who postulated that the presence of “sprouting” at the invasive edge of carcinomas

reflected a more rapid tumor growth rate.⁽³⁾ Although a large number of studies have shown that tumor budding/sprouting is an independent adverse prognostic factor in colorectal carcinoma, assessment methods and definitions have varied widely between studies.⁽³⁾ It is sometimes difficult to detect budding foci by conventional pathological examination of H&E stained sections alone because tumor buds can be very small and resemble the surrounding stromal cells.^(3,6) Furthermore, the distribution and frequency of budding foci differs even among tumors with the same budding grade.

The molecular mechanisms underlying tumor budding/sprouting remain unclear. In this study, we focused on the role of c-Met in budding tumor cells. The c-Met protein is an important factor in colorectal tumorigenesis, progression, and metastasis.⁽⁷⁾ The *MET* gene is located on chromosome 7 at q31 and encodes a transmembrane glycoprotein that serves as a specific receptor for hepatocyte growth factor (HGF).⁽⁸⁾ Binding of HGF to c-Met induces phosphorylation of tyrosine residues at the C-terminus of the receptor, leading to receptor activation.⁽⁹⁾ Hepatocyte growth factor/MET signaling pro-

motes multiple biological activities, including cell proliferation, motility, invasion, angiogenesis, and morphogenesis in a wide variety of normal and neoplastic cells.⁽¹⁰⁾ Moreover, c-Met activity is deregulated in many human cancers, including colorectal carcinoma, as a result of genetic mutations, gene amplification, protein overexpression, or production of HGF-dependent autocrine circuits.^(11,12) In colorectal carcinoma, increased expression of the c-Met protein is associated with highly invasive tumors that spread through the intestinal wall.^(8,13)

Our study had two major aims: (i) to evaluate the associations between our scoring system for tumor budding/sprouting, which included budding grade and the proportion of budding-positive areas, and clinicopathologic factors or prognosis; and (ii) to assess the association between c-Met expression and tumor budding/sprouting. Assessment of the budding score was significantly associated with lymphovascular invasion, lymph node (LN) metastasis, and poor prognosis. Moreover, we found a significant correlation between c-Met expression levels at the invasive tumor front and budding score.

Materials and Methods

Patients. We retrospectively reviewed 139 patients who underwent surgical resection of primary colorectal adenocarcinomas at the Department of Gastroenterological Surgery,

Fukuoka University Hospital (Fukuoka, Japan) from January 2005 to December 2009. Patients with familial adenomatous polyposis, hereditary non-polyposis colorectal cancer syndrome, or inflammatory bowel disease were excluded. Tissues from surgical resections can be used for research according to the standard treatment agreement with patients in our hospital, provided patient anonymity is maintained and the patient has no objections. The protocol for this study was approved by the Institutional Review Board (Ethics Committee). Pathologic stage and tumor differentiation were determined by the TNM classification of malignant tumors (Union for International Cancer Control) and the Japanese Classification of Colorectal Carcinoma (JCCC),⁽¹⁴⁾ respectively. Complete tumor resection was achieved in 114 cases, including 10 cases of pTis tumors for which endoscopic treatment could not be carried out. None of the patients received preoperative radiotherapy or chemotherapy.

Tissue samples and immunohistochemistry (IHC). Surgically resected specimens were fixed in 10% formalin and processed into paraffin blocks. Tissues were sectioned (3- μ m thickness), deparaffinized, and immersed in 0.3% hydrogen peroxide in methanol for 10 min at room temperature to block endogenous peroxidase activity. For anti-cytokeratin (CK) antibody staining, sections were heated in 10 mM EDTA buffer (pH 8.0) in a microwave oven (700 W) for 10 min to retrieve epitopes. After non-specific sites were blocked with Serum-Free Protein Block (Dako, Carpinteria,

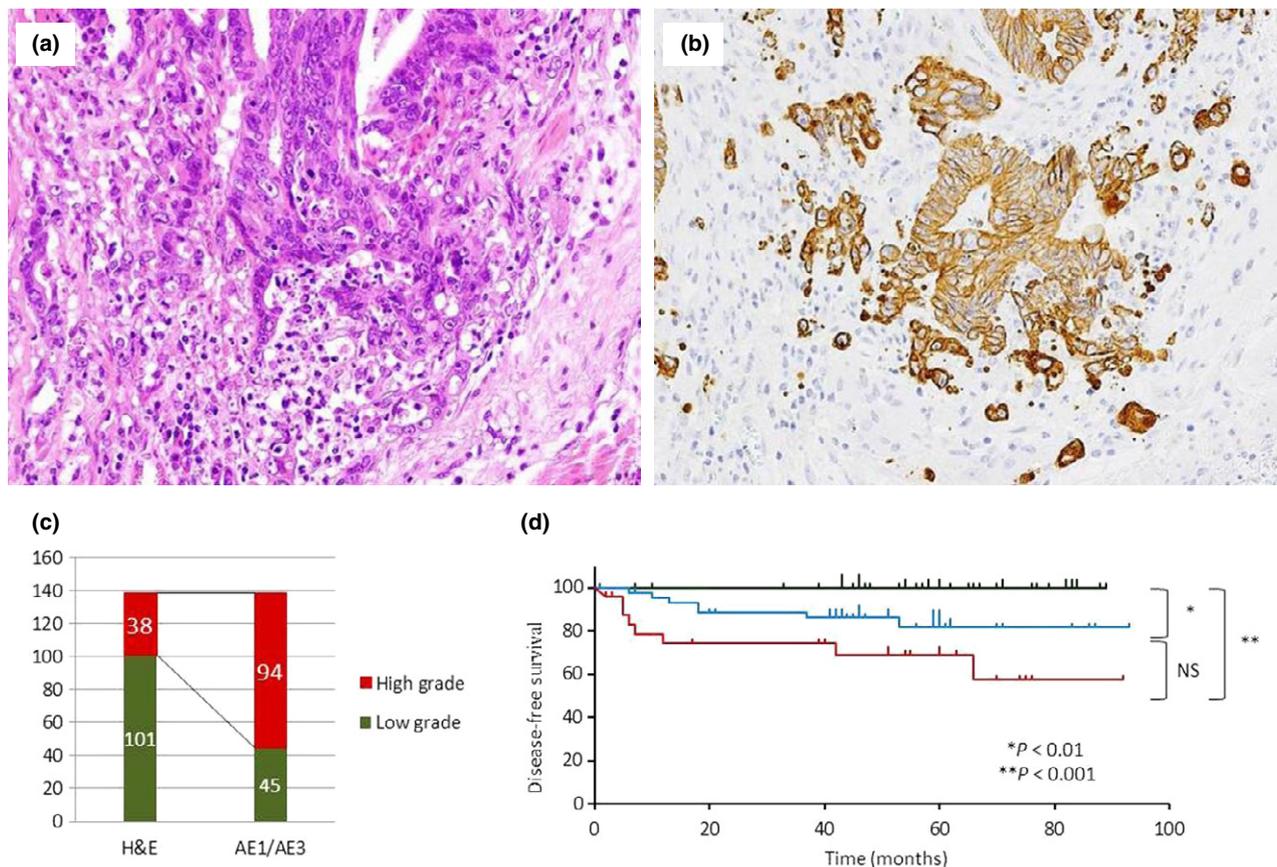


Fig. 1. Hematoxylin–eosin staining of colorectal cancer showing tumor budding/sprouting (a) and cytokeratin immunohistochemistry (CK-IHC) for tumor budding/sprouting (b) at the invasive front. (c) Classification of budding grade by H&E staining and CK-IHC. (d) Disease-free survival curves for patients with different budding grades assessed by H&E and CK-IHC. Green line, 40 tumors assessed as low grade budding with both H&E and CK-IHC; blue line, 47 tumors assessed as low grade with H&E but high grade with CK-IHC; red line, 26 tumors assessed as high grade with both H&E and CK-IHC. NS, not significant.

CA, USA) for 30 min at room temperature, sections were incubated with antibodies against CK (AE1/AE3) (dilution 1:200; Dako), c-Met (dilution 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or phosphorylated c-Met (p-c-Met) (dilution 1:1,000; Immuno-Biological Laboratories, Takasaki, Japan) for 1 h at room temperature (AE1/AE3) or overnight at 4°C (c-Met, p-c-Met). Sections were then washed and incubated with ChemMate EnVision (Dako). Immunoreactive proteins were visualized with 3,3'-diaminobenzidine (Dako), followed by counterstaining with hematoxylin. A diagnosis was independently made by two pathologists and confirmed when the two opinions were concordant.

Scoring system for tumor budding/sprouting. Based on JCCC,⁽¹⁴⁾ we defined tumor budding/sprouting as a cancer cell nest consisting of five or fewer cells that infiltrated the interstitium at the invasive margin of the cancer (Fig. 1). After select-

ing an area in which budding/sprouting was most intensive, the buds were counted in a field measuring 0.785 mm² through a 20 × objective lens (WHK 10 × ocular lens; Olympus, Tokyo, Japan). Depending on the number of buds, the criteria by JCCC for estimation of tumor budding/sprouting were modified as follows: grade 0, no budding; grade 1, 1–4 buds; grade 2, 5–9 buds; and grade 3, 10 or more buds. We used this grading system to evaluate budding/sprouting in H&E- and CK-immunostained sections. The extent of budding/sprouting was classified as low grade (grades 0 and 1) or high grade (grades 2 and 3).

The distribution of budding foci differed, even among tumors of the same grade (Fig. 2a,b). For example, in some tumors only a few grade 2 budding/sprouting foci were present whereas other tumors showed grade 2 budding foci at higher frequencies along the invasive front. Therefore, we assessed both budding

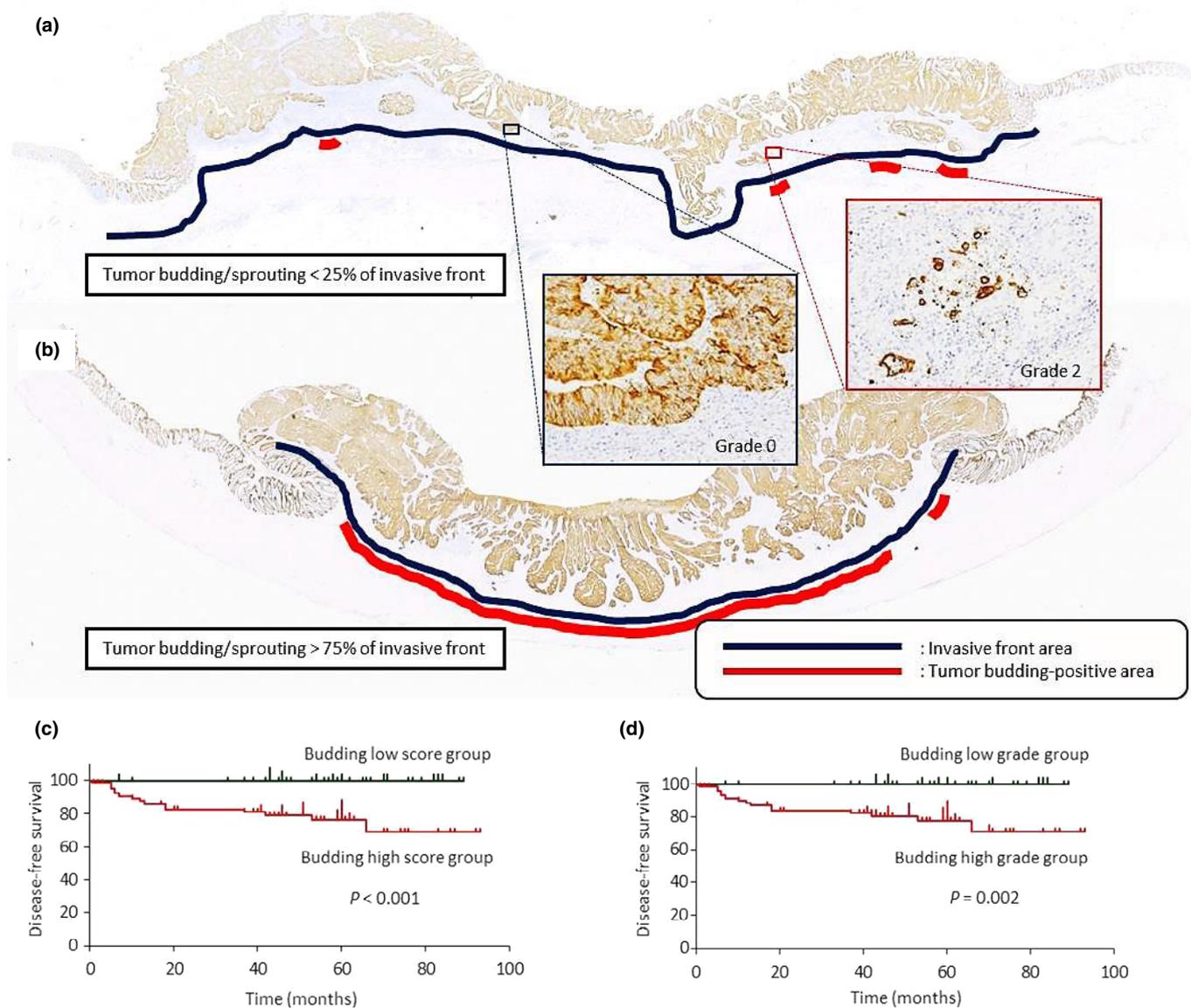


Fig. 2. Score for colorectal cancer budding/sprouting based on grade and area. (a,b) Both of these tumors were categorized as budding grade 2, but the area of the invasive front positive for tumor budding/sprouting was different. (c,d) Disease-free survival (DFS) curves stratified by budding score and grade for 114 patients who underwent complete resection of colorectal tumors. (c) DFS curves for patients with high and low budding score were significantly different ($P < 0.001$). (d) DFS curves for patients with high and low budding grade assessed by cytokeratin immunohistochemistry were significantly different ($P = 0.002$).

grade and the proportion of budding-positive areas. Each budding grade (grade 0–3 by CK-IHC) was multiplied by an area score (0.25, 0–25%; 0.5, 26–50%; 0.75, 51–75%; and 1, 76–100%), giving a maximum total score of 3. This budding score was classified as low (<1) or high (≥1).

Scoring for c-Met and p-c-Met. Immunoreactivity for c-Met or p-c-Met was observed as cytoplasmic or membrane staining in carcinoma cells. Staining results were assessed semiquantitatively with the modified McCarty's H-scoring system.⁽¹⁵⁾ The intensity score (0, none; 1, weak; 2, moderate; and 3, strong) and proportion score (0, 0–5%; 1, 6–25%; 2, 26–50%; 3, 51–75%; and 4, 76–100%) were multiplied to yield an overall c-Met or p-c-Met score. These c-Met and p-c-Met scores were classified as low or high corresponding to overall scores of 0–4 or 6–12, respectively.

Fluorescence *in situ* hybridization analysis. Fluorescence *in situ* hybridization assays were carried out at the invasive fronts and within superficial portions of tumors for 21 or 22 randomly selected recent cases of budding/sprouting with low or high score, respectively. The *MET* gene was evaluated on formalin-fixed paraffin-embedded 4- μ m thick tissue sections using Vysis LSI *MET/CEP 7* probe (Abbott, Tokyo, Japan) with slight modifications, as described previously.⁽¹⁶⁾ At least 60 cells were scored for each case.

The copy number of the *MET* gene was classified into six categories (disomy, low and high trisomy, low and high polyploidy, and gene amplification) according to the number of copies of the *MET* gene and chromosome 7 centromere.⁽¹⁰⁾ The mean *MET* gene copy number per cell was also recorded for each case.

Statistical analysis. The relationships between several clinicopathologic parameters and the results of budding assessment, FISH, and IHC were evaluated with the chi-square-test and Fisher's exact test. The results of IHC and FISH analyses were compared using the Mann–Whitney *U*-test at both the invasive front and superficial portions of colorectal cancers. Survival curves were plotted by the Kaplan–Meier method, and *P*-values were calculated with the log–rank test. A *P*-value <0.05 was considered statistically significant. All data analyses were carried out using StatMate IV for Windows (ATMS, Tokyo, Japan).

Results

Clinicopathologic findings and budding grades by H&E staining or CK-IHC. Table 1 summarizes the clinicopathologic characteristics of the 139 patients (84 males and 55 females; age range, 25–94 years [mean, 66.7]). The median follow-up period was 40.5 months (range, 0–93).

Tumor budding/sprouting was first assessed in H&E stained sections. However, assessment was sometimes difficult because of the presence of reactive fibroblasts or macrophages in the stroma surrounding the invasive front, therefore we investigated whether it was easier to detect budding carcinoma cells with CK-IHC. Among 139 tumors, H&E revealed low- and high-grade budding in 101 (72.7%) and 38 (27.3%) tumors, respectively, whereas CK-IHC showed low- and high-grade budding in 45 (32.4%) and 94 (67.6%) tumors, respectively (Fig. 1a–c). Cytokeratin–IHC revealed approximately 2.5-fold more cases of high-grade budding than H&E staining. Comparing the two assessment methods, 37 of 38 tumors that showed high-grade budding with H&E remained high by CK-IHC (high-to-high group). In contrast, only 44 of 101 tumors that showed low-grade

Table 1. Clinicopathological features of 139 patients with surgically resected primary colorectal adenocarcinomas

	<i>n</i>	%
Patients	139	na
Gender		
Male	84	60.4
Female	55	39.6
Age, years		
Mean	66.7	na
Range	25–94	na
Location		
Cecum	8	5.8
Ascending colon	21	15.1
Transverse colon	15	10.8
Descending colon	7	5.0
Sigmoid colon	30	21.6
Rectum	58	41.7
Tumor differentiation		
Well	63	45.3
Moderately	75	54.0
Poorly	1	0.7
Wall penetration (pT)		
pTis	10	7.2
pT1	27	19.4
pT2	21	15.1
pT3	47	33.8
pT4	34	24.5
Lymphatic invasion		
Present	80	57.6
Absent	59	42.4
Venous invasion		
Present	103	74.1
Absent	36	25.9
Lymph node metastasis		
Present	50	36.0
Absent	89	64.0
TNM stage		
0	9	6.5
I	42	30.2
II	29	20.9
III	35	25.2
IV	24	17.3

na, not applicable.

budding with H&E remained low grade with CK-IHC (low-to-low group) and 57 were classified as high-grade (low-to-high group). Both H&E- and CK-IHC-based grades showed significant association with all three parameters, lymphatic and venous invasion and lymph node metastasis (Table 2). However, the high grade of the CK-IHC budding grade detected more cases with lymphatic and venous invasion and lymph node metastasis than that of the H&E budding grade (80% vs. 41%, 78% vs. 34%, and 96% vs. 54%, respectively), indicating usefulness of the CK-IHC grade. When disease-free survival (DFS) was assessed in 113 patients who received curative resections (40 low-to-low, 47 low-to-high, and 26 high-to-high), the low-to-high group (Fig. 1d, blue line) showed an intermediate survival curve between that for the low-to-low (green line) and high-to-high (red line) groups. The differences between low-to-low and low-to-high and between low-to-low

Table 2. Clinicopathological findings in relation to budding grade of primary colorectal adenocarcinomas by H&E staining and immunohistochemistry (IHC)

	Budding grade (H&E)			Budding grade (IHC)		
	Low grade	High grade	<i>P</i> -value	Low grade	High grade	<i>P</i> -value
Lymphatic invasion						
Present	47	33	<0.001	9	71	<0.001
Absent	54	5		36	23	
Venous invasion						
Present	68	35	<0.01	23	80	<0.001
Absent	33	3		22	14	
Lymph node metastasis						
Present	23	27	<0.001	2	48	<0.001
Absent	78	11		43	46	

The degree of budding/sprouting was classified as low grade or high grade corresponding to 0–4 (grade 0 and grade 1) and ≥ 5 budding foci (grade 2 and grade 3) in one field, respectively.

and high-to-high groups were statistically significant. The presence of the low-to-high group, which showed survival in between the low-to-low and high-to-high group, also supported the clinicopathological significance of the CK-IHC grade.

Scoring for tumor budding/sprouting. Next, we considered budding-positive areas in our assessment of tumor budding/sprouting (Fig. 2a,b) and identified 51 (36.7%) low-score and 88 (63.3%) high-score tumors. A high score for budding-positive areas was significantly associated with greater wall penetration, increased lymphovascular invasion, and more frequent lymph node metastasis (Table 3).

The association between budding score or grade and DFS was assessed in 114 patients who received curative resections. Disease-free survival was significantly shorter in patients with high budding scores or grades compared with that in patients with low budding scores or grades, respectively ($P < 0.001$ for budding scores and $P = 0.002$ for budding grade) (Fig. 2c). Thus, both budding score and grade revealed significant association with DFS. In this study, we used the budding score for analyses of budding mechanisms as tumor budding is assessed more precisely by budding grade and areas, although budding grade alone is more practical for daily diagnosis.

Scores for c-Met and p-c-Met at different tumor sites. We examined the relationship between the expression of c-Met or p-c-Met and the extent of tumor budding/sprouting to assess the role of c-Met signaling in tumor budding/sprouting. Carcinoma cells including budding cells expressed c-Met and p-c-Met in the cytoplasm and cell membrane, as reported in other tumors.^(17,18) Representative images of different intensity scores for c-Met and p-c-Met are shown in Figure 3.

Tumors with both low and high scores for tumor budding/sprouting showed significantly higher c-Met scores at the invasive front than in superficial portions of the tumors. Moreover, c-Met scores at the invasive front were significantly higher in tumors with high budding scores than those in tumors with low budding scores ($P < 0.05$). In contrast, c-Met scores in superficial portions were not significantly different between tumors with low and high scores for tumor budding/sprouting ($P = 0.32$) (Fig. 4a).

Immunohistochemistry for p-c-Met showed similar results as staining for c-Met, except that p-c-Met scores were not significantly different between the invasive front and superficial portions of tumors with low-score budding (Fig. 4b). Tumor cells expressing c-Met and p-c-Met were observed

Table 3. Clinicopathological findings in relation to budding score in primary colorectal adenocarcinomas

	Budding score		<i>P</i> -value
	Low score (score <1) (<i>n</i> = 51)	High score (score ≥ 1) (<i>n</i> = 88)	
Tumor size			
<5 cm	38	59	0.360
≥ 5 cm	13	29	
pT-stage			
pTis, pT1, pT2	33	25	<0.001
pT3, pT4	18	63	
Lymphatic invasion			
Present	14	66	<0.001
Absent	37	22	
Venous invasion			
Present	28	75	<0.001
Absent	23	13	
Lymph node metastasis			
Present	6	44	<0.001
Absent	45	44	

more frequently in budding cells and tumor cell nests at or near the invasion front than superficial portions of tumors with high budding scores. This difference in distribution of p-c-Met-expressing cells was not evident in tumors with low budding scores.

Fluorescence *in situ* hybridization analysis. The FISH analysis for *MET* copy number showed occasional trisomy, polysomy, and disomy (Fig. 5a). Amplification was not observed in this study ($n = 43$ tumors). The *MET* gene copy number per cell was significantly higher at the invasive front than in the superficial portion of tumors in tumors with a high budding score. However, the copy number was less than 2.5 even at the invasive front of tumors with high-score budding, therefore no gene amplification was detected (Fig. 5b).

Univariate analysis. Among 114 tumors, univariate analysis identified significant associations between shorter DFS and budding score (high score), budding grades (high grades), lymphatic invasion (positive), venous invasion (positive), LN metastasis (positive), tumor size (≥ 5 cm), pathologic wall

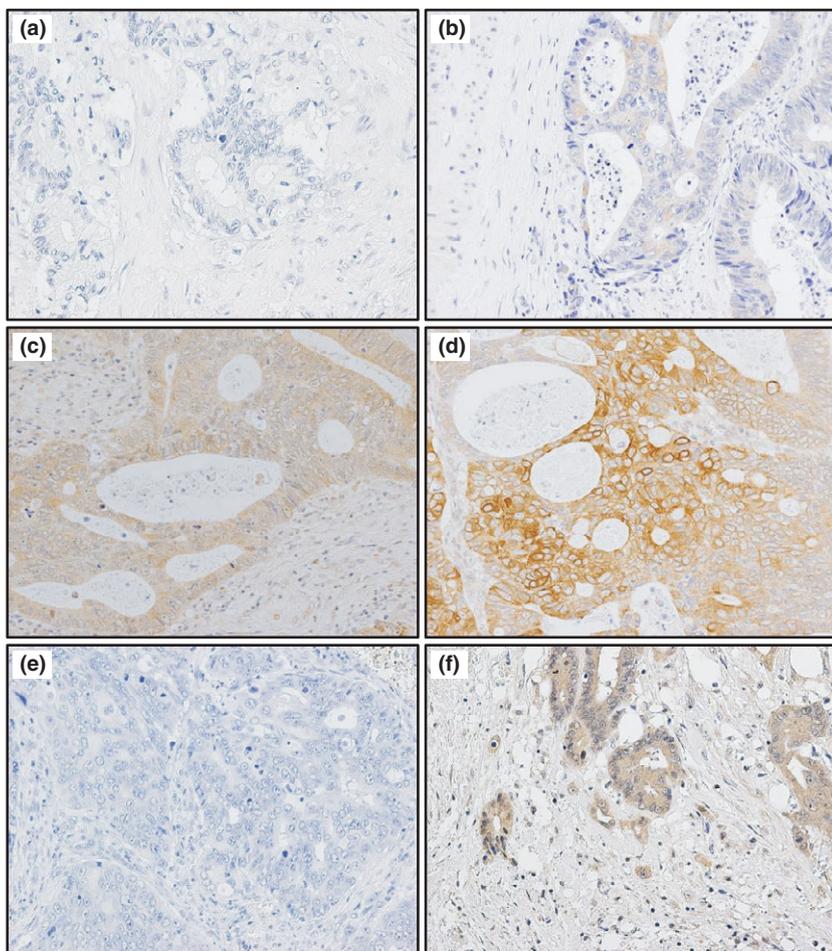


Fig. 3. Representative images of c-Met expression in tissue sections of colorectal cancer by immunohistochemistry. (a) No c-Met expression in tumor cells (scored as 0). (b) Weak c-Met expression in tumor cells (scored as 1). (c) Moderate c-Met expression in tumor cells (scored as 2). (d) Strong c-Met expression in tumor cells forming nests near the invasive front (scored as 3). (e) No phosphorylated c-Met expression in tumor cells in superficial portion (scored as 0). (f) Moderate phosphorylated c-Met expression in tumor cells including budding cells at the invasive front (scored as 2).

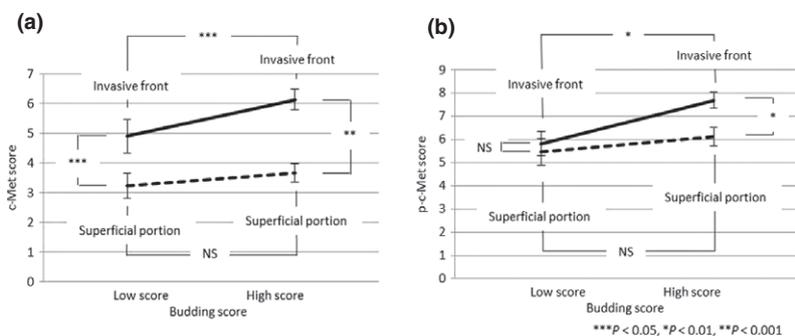


Fig. 4. Comparison of c-Met (a) and phosphorylated c-Met (p-c-Met) scores (b) in different colorectal cancer tumor regions between tumors with high and low budding scores. NS, not significant.

penetration (T3 and T4), p-c-Met score (high score), and *MET* gene copy number per cell (≥ 2.4) (Table 4).

Discussion

This study has shown that tumor budding/sprouting assessed by grade and area shows a strong correlation with clinicopathologically aggressive parameters in colorectal carcinoma. Although our assessment may not be useful for routine clinical practice, it is appropriate for examining the molecular mechanisms of tumor budding/sprouting. This is the first sug-

gestion of a potential association between c-Met activation and formation of tumor budding/sprouting at the invasive front.

Morodomi *et al.*⁽¹⁹⁾ and Ueno *et al.*⁽²⁰⁾ provided budding grades that can be used routinely for histopathologic diagnosis by examination of H&E sections. However, these grades have several limitations regarding objective assessment of tumor budding/sprouting. In some cases, tumor buds cannot be detected easily with H&E staining alone because stromal reaction, such as aggregation of active fibroblasts and/or histiocytes, is present at the invasive front and often masks the

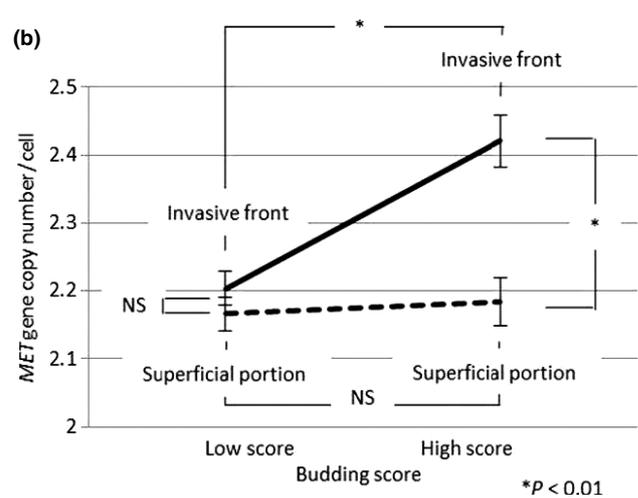
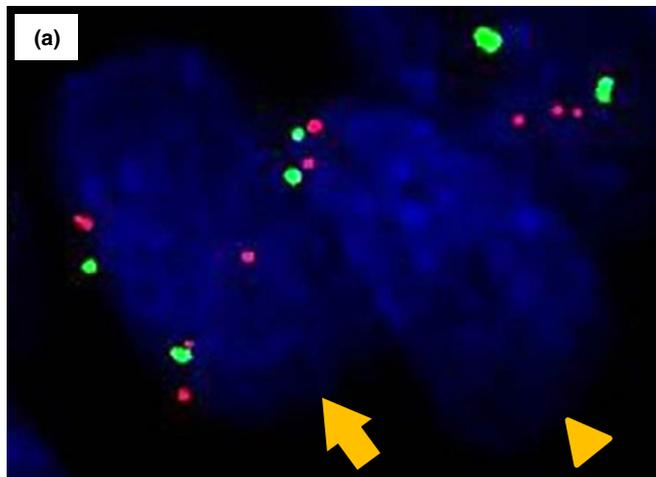


Fig. 5. (a) Dual-color FISH assays with probes for *MET* (red) and chromosome 7 centromere (green) in colorectal cancer tissue. The *MET* gene copy number in the cells shown was two (arrowhead) and four (arrow). (b) Comparison of *MET* gene copy number per cell by FISH in different tumor regions between tumors with high and low budding scores. NS, not significant.

Table 4. Results of univariate analysis on postoperative disease-free survival in patients with surgically resected primary colorectal adenocarcinomas

Factor	Disease-free survival (n = 114)		P-value
	n		
Budding grade (IHC)	Low grade	41	0.002
	High grade	73	
Budding score	Low score	46	<0.001
	High score	68	
Lymphatic invasion	Present	58	0.010
	Absent	56	
Venous invasion	Present	78	0.024
	Absent	36	
Lymph node metastasis	Present	35	0.006
	Absent	79	
Tumor size	<5 cm	86	<0.001
	≥5 cm	28	
Wall penetration (pT)	Tis, T1, T2	57	<0.001
	T3, T4	57	
c-Met score	Low score	47	0.670
	High score	67	
p-c-Met score	Low score	35	0.030
	High score	79	
<i>MET</i> gene copy number per cell	<2.4	24	0.140
	≥2.4	11	

Scores of c-Met and phosphorylated c-Met (p-c-Met) were classified as low or high corresponding to 0–4 and 6–12, respectively. IHC, immunohistochemistry.

buds. Also, marked inflammation can obscure budding tumor cells.⁽³⁾ Therefore, as in previous studies,^(2,6,21–25) we assessed tumor budding/sprouting by IHC with anti-CK antibody to more accurately and objectively diagnose tumor budding/sprouting. We found that CK-IHC was very useful for the detection of single cancer cells or small cancer cell clusters embedded in fibrous stroma. One previous study⁽²⁵⁾ that included both H&E and CK-IHC showed a modest improvement in inter-observer variability with the latter.⁽³⁾ We

compared budding counts obtained with H&E to those obtained with CK-IHC and obtained significantly higher counts with the latter. Moreover, CK-IHC could identify tumors with shorter DFS among tumors assessed as low-grade budding by H&E alone. Thus, CK-IHC was useful for objective evaluation of budding grade.

In our study, the frequency of tumor budding/sprouting along the entire invasive front differed, even among tumors with the same budding grade. The majority of previous studies evaluated the budding grade only at the invasive front with the greatest amount of tumor budding/sprouting, although a few studies⁽²⁶⁾ also evaluated the proportion of budding-positive areas. The latest study by Horcic *et al.*⁽²⁷⁾ reported that one visual field is suboptimal to assess tumor budding because of heterogeneous biological features in CRC. Tumor buds are not homogeneously distributed along the invasive border. Therefore, Horcic *et al.* recommends that tumor budding assessment should be carried out using 10 high power fields to account for the biological variation within a histological section. We assessed tumor budding variation in terms of the frequency of budding along the invasive border instead of assessing 10 high power fields. In this way, we developed an original scoring system that includes both budding grade and area. A high budding score using our system was significantly correlated with a shorter DFS, and this correlation was slightly more significant than that obtained by assessing budding grade alone. These results suggest that the budding score is a predictive factor for recurrence after curative resection.

Previous studies have shown positive staining for c-Met by IHC in 60–79% of colorectal carcinomas^(28,29) and *MET* gene amplification by FISH in 2.6% of carcinomas.⁽³⁰⁾ However, the relationship between c-Met and tumor budding/sprouting has not been explored fully. In this study, the expression and phosphorylation levels of c-Met were significantly higher at the invasive fronts than in superficial portions of tumors in the high-score budding group. No *MET* gene amplification was detected, although a significant increase in the *MET* gene copy number per cell was seen at the invasive front of the high-score budding group. Metastatic colon carcinoma tissues in the liver showed the gene copy number similar to that at the invasive front ($n = 3$, 2.39 ± 0.16 ; preliminary study, data not shown). However, the gene copy numbers were less than 2.5 in both

metastatic liver tumors and invasive fronts of tumors with high-score budding, and thus their biological or clinical significance in c-Met expression is still unknown and needs to be clarified in the future. Therefore, in this study, we consider that post-transcriptional activation of c-Met by phosphorylation may be involved in tumor budding/sprouting at invasive fronts. This phosphorylation of c-Met may be induced by HGF produced by stromal cells. In colorectal carcinoma, HGF was actually detected in mesenchymal cells in association with c-Met expression in carcinoma cells.⁽³¹⁾ A previous study showed that c-Met was expressed in tumor cells and stromal HGF at a significantly higher level in type C invasive tumors than in type A and B non-invasive tumors based on Noguchi's classification for lung adenocarcinomas.⁽¹⁰⁾ In another study using 3-D co-cultures of a ductal breast carcinoma *in situ* cell line and HGF-secreting fibroblasts, fibroblast-derived HGF promoted progression of *in situ* ductal carcinoma to invasive carcinoma.⁽³²⁾ These results indicate that HGF produced by active fibroblasts might be one of the factors triggering the transition of non-invasive tumors to invasive adenocarcinomas. Therefore, the expression and phosphorylation of c-Met at tumor invasive fronts observed in our study may be related to tumor–stroma interactions. Furthermore, the presence of more p-c-Met expressing cells at the invasion front in the high-score budding group suggested an association between the activated c-Met signaling pathway and tumor budding/sprouting. Activation of c-Met in tumors leads to cell proliferation, angiogenesis, invasion, and cell migration, including cell scattering.^(10–12) The

presence of tumor buds is considered to represent epithelial–mesenchymal transition,⁽³³⁾ a process frequently associated with increased expressions of molecules relevant to tumor invasion, such as MMPs, β -catenin, and laminin 5 γ 2 in tumor cells, and stimulation of the Wnt signaling pathway.⁽³³⁾ In our study, p-c-Met expression was frequently observed not only in tumor buds but also in larger tumor nests near the invasion front, suggesting that additional factor/s may be needed for cells undergoing tumor budding/sprouting. Our finding that the p-c-Met score was associated with postoperative recurrence is also of potential clinical significance. Future studies should examine whether c-Met inhibition reduces the invasiveness or recurrence of colorectal carcinoma.

Acknowledgments

We thank Ms. M. Onitsuka, Ms. K. Yano, Ms. T. Nakamura, and Mr. S. Matsumoto for excellent technical assistance in IHC and FISH. This work was supported in part by grants from the Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology (No. 24590495) and the Research Center for Advanced Molecular Medicine, Fukuoka University.

Disclosure Statement

The authors have no conflict of interest.

References

- Suzuki H, Masuda N, Shimura T *et al.* Nuclear beta-catenin expression at the invasive front and in the vessels predicts liver metastasis in colorectal carcinoma. *Anticancer Res* 2008; **28**: 1821–30.
- Prall F, Nizze H, Barten M. Tumour budding as prognostic factor in stage I/II colorectal carcinoma. *Histopathology* 2005; **47**: 17–24.
- Mitrovic B, Schaeffer DF, Riddell RH, Kirsch R. Tumor budding in colorectal carcinoma: time to take notice. *Mod Pathol* 2012; **25**: 1315–25.
- Park KJ, Choi HJ, Roh MS, Kwon HC, Kim C. Intensity of tumor budding and its prognostic implications in invasive colon carcinoma. *Dis Colon Rectum* 2005; **48**: 1597–602.
- Puppa G, Senore C, Sheahan K *et al.* Diagnostic reproducibility of tumour budding in colorectal cancer: a multicentre, multinational study using virtual microscopy. *Histopathology* 2012; **61**: 562–75.
- Kazama S, Watanabe T, Ajioka Y, Kanazawa T, Nagawa H. Tumour budding at the deepest invasive margin correlates with lymph node metastasis in submucosal colorectal cancer detected by anticytokeratin antibody CAM5.2. *Br J Cancer* 2006; **94**: 293–8.
- Maulik G, Shrikhande A, Kijima T, Ma PC, Morrison PT, Salgia R. Role of the hepatocyte growth factor receptor, c-Met, in oncogenesis and potential for therapeutic inhibition. *Cytokine Growth Factor Rev* 2002; **13**: 41–59.
- Zeng ZS, Weiser MR, Kuntz E *et al.* c-Met gene amplification is associated with advanced stage colorectal cancer and liver metastases. *Cancer Lett* 2008; **265**: 258–69.
- Janjigian YY, Tang LH, Coit DG *et al.* MET expression and amplification in patients with localized gastric cancer. *Cancer Epidemiol Biomarkers Prev* 2011; **20**: 1021–7.
- Tachibana K, Minami Y, Shiba-Ishii A *et al.* Abnormality of the hepatocyte growth factor/MET pathway in pulmonary adenocarcinogenesis. *Lung Cancer* 2012; **75**: 181–8.
- Trusolino L, Comoglio PM. Scatter-factor and semaphorin receptors: cell signalling for invasive growth. *Nat Rev Cancer* 2002; **2**: 289–300.
- Wielenga VJ, van der Voort R, Taher TE *et al.* Expression of c-Met and heparan-sulfate proteoglycan forms of CD44 in colorectal cancer. *Am J Pathol* 2000; **157**: 1563–73.
- Takeuchi H, Bilchik A, Saha S *et al.* c-MET expression level in primary colon cancer: a predictor of tumor invasion and lymph node metastases. *Clin Cancer Res* 2003; **9**: 1480–8.
- Rectum JSfCotCa. *Japanese Classification of Colorectal carcinoma*, 8th edn. Tokyo: Kanehara, 2013.
- McCarty KS Jr, Miller LS, Cox EB, Konrath J, McCarty KS Sr. Estrogen receptor analyses. Correlation of biochemical and immunohistochemical methods using monoclonal antireceptor antibodies. *Arch Pathol Lab Med* 1985; **109**: 716–21.
- Matsumoto S, Nabeshima K, Kamei T *et al.* Morphology of 9p21 homozygous deletion-positive pleural mesothelioma cells analyzed using fluorescence *in situ* hybridization and virtual microscope system in effusion cytology. *Cancer Cytopathol* 2013; **121**: 415–22.
- Koga K, Hamasaki M, Kato F *et al.* Association of c-Met phosphorylation with micropapillary pattern and small cluster invasion in pT1-size lung adenocarcinoma. *Lung Cancer* 2013; **82**: 413–9.
- Moschetta M, Basile A, Ferrucci A *et al.* Novel targeting of phospho-cMET overcomes drug resistance and induces antitumor activity in multiple myeloma. *Clin Cancer Res* 2013; **19**: 4371–82.
- Morodomi T, Isomoto H, Shirouzu K, Kakegawa K, Irie K, Morimatsu M. An index for estimating the probability of lymph node metastasis in rectal cancers. Lymph node metastasis and the histopathology of actively invasive regions of cancer. *Cancer* 1989; **63**: 539–43.
- Ueno H, Murphy J, Jass JR, Mochizuki H, Talbot IC. Tumour 'budding' as an index to estimate the potential of aggressiveness in rectal cancer. *Histopathology* 2002; **40**: 127–32.
- Ohtsuki K, Koyama F, Tamura T *et al.* Prognostic value of immunohistochemical analysis of tumor budding in colorectal carcinoma. *Anticancer Res* 2008; **28**: 1831–6.
- Shinto E, Jass JR, Tsuda H *et al.* Differential prognostic significance of morphologic invasive markers in colorectal cancer: tumor budding and cytoplasmic podia. *Dis Colon Rectum* 2006; **49**: 1422–30.
- Shinto E, Mochizuki H, Ueno H, Matsubara O, Jass JR. A novel classification of tumour budding in colorectal cancer based on the presence of cytoplasmic pseudo-fragments around budding foci. *Histopathology* 2005; **47**: 25–31.
- Losi L, Ponti G, Gregorio CD *et al.* Prognostic significance of histological features and biological parameters in stage I (pT1 and pT2) colorectal adenocarcinoma. *Pathol Res Pract* 2006; **202**: 663–70.
- Suzuki A, Togashi K, Nokubi M *et al.* Evaluation of venous invasion by Elasticin van Gieson stain and tumor budding predicts local and distant metastases in patients with T1 stage colorectal cancer. *Am J Surg Pathol* 2009; **33**: 1601–7.
- Nakamura T, Mitomi H, Kanazawa H, Ohkura Y, Watanabe M. Tumor budding as an index to identify high-risk patients with stage II colon cancer. *Dis Colon Rectum* 2008; **51**: 568–72.

- 27 Horcic M, Koelzer VH, Karamitopoulou E *et al.* Tumor budding score based on 10 high-power fields is a promising basis for a standardized prognostic scoring system in stage II colorectal cancer. *Hum Pathol* 2013; **44**: 697–705.
- 28 Abou-Bakr AA, Elbasmi A. c-MET overexpression as a prognostic biomarker in colorectal adenocarcinoma. *Gulf J Oncolog* 2013; **1**: 28–34.
- 29 De Oliveira AT, Matos D, Logullo AF *et al.* MET Is highly expressed in advanced stages of colorectal cancer and indicates worse prognosis and mortality. *Anticancer Res* 2009; **29**: 4807–11.
- 30 Cappuzzo F, Varella-Garcia M, Finocchiaro G *et al.* Primary resistance to cetuximab therapy in EGFR FISH-positive colorectal cancer patients. *Br J Cancer* 2008; **99**: 83–9.
- 31 Otte JM, Schmitz F, Kiehne K *et al.* Functional expression of HGF and its receptor in human colorectal cancer. *Digestion* 2000; **61**: 237–46.
- 32 Jedezsko C, Victor BC, Podgorski I, Sloane BF. Fibroblast hepatocyte growth factor promotes invasion of human mammary ductal carcinoma *in situ*. *Cancer Res* 2009; **69**: 9148–55.
- 33 Zlobec I, Lugli A. Epithelial mesenchymal transition and tumor budding in aggressive colorectal cancer: tumor budding as oncotarget. *Oncotarget* 2010; **1**: 651–61.